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Comparisons of metabolic and physiological changes in rats following short term oral dosing with pesticides commonly found in food

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Suggested Running Title: Metabolomics for food toxicology assessment

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Abstract

^{1}H Nuclear Magnetic Resonance spectroscopy has been used to profile urinary metabolites in male Fischer F344 rats in order to assess the metabolic changes induced by oral exposure to two benzimidazole fungicides (carbendazim and thiabendazole) and two bipyridyllium herbicides (chlormequat and mepiquat). Exposure levels were selected to be lower than those expected to cause overt signs of toxicity. We then compared the sensitivity of the metabolomics approach to more traditional methods of toxicity assessment such as the measurement of growth and organ weights. Separate, acute exposure experiments were conducted for each pesticide to identify potential metabolic markers of exposure across four doses (and a control). Growth, organ weights and feeding/drinking rates were not significantly affected by any compounds at any dose levels tested. In contrast, metabolic responses were detected within 8 and 24 hours for chlormequat and mepiquat, and after 24 hours for carbendazim and thiabendazole. These results demonstrate the potential for the use of metabolomics in food toxicity testing.

Keywords

Food safety; metabolomics; toxicology; time course

1. Introduction

The majority of crops grown worldwide are routinely treated with a variety of pesticides throughout the growing season. The extent of pesticide usage varies with each crop and between countries but many compound classes are applied throughout the year. For example, herbicides are applied on arable crops in the UK with an average of three applications of four products and five active substances per year (Garthwaite et al., 1999). This leads to pesticide residues occurring in food products, albeit at low concentrations of each individual compound (Hernández-Borges et al., 2009). In recent years the increasing public concern over this issue has led to the need for tools to aid in the hazard identification and risk assessment of these chemicals to be clearly recognized (Miles and Frewer, 2001; Royal Commission on Environmental Pollution, 2003).

The risk posed to human health from pesticide residues in foodstuffs is currently assessed on a compound by compound basis using residue data from environmental monitoring in conjunction with toxicological information obtained from laboratory studies on model species (Bondy et al., 2004). However, these assessments tend to be aimed at finding the level that causes gross histopathological changes, such as organ damage, behavioural problems (such as the blinking response) or a reduction in feeding/drinking rates. These studies are clearly important but by focussing on overt toxicity they may miss subtle, low level effects on metabolism. Such metabolic changes may not directly reflect those seen at high levels of exposure (food toxicity) of individual pesticides since low levels of toxicant exposure may induce different changes than those induced by higher exposures but they have could be used to provide early warnings of possible toxicity prior to irreversible damage

occurring; this would have great potential use in food safety assessment and regulation (Favé et al., 2009).

Metabolomics provides a quick and convenient technique to investigate potential toxicity in biofluids from any species (Griffin and Shore, 2007). The approach is particularly useful at confirming organ specific toxicity following the identification of metabolic markers in biofluids (Beckwith-Hall et al., 1998; Garrod et al., 2005). This method also has a large practical advantage over other omic/systems biology based technologies such as transcriptomics and proteomics in that metabolites are similar in the majority of species; thus, a fully annotated genome is not required for analysis and analytical methods are transferable between species (Jones et al., 2008). Here, we have used ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy to analyse metabolic profiles from urine collected from rats (*Rattus norvegicus* – inbred Fischer F344 strain) exposed to single doses of one of four different, yet commonly used, pesticides which have been shown to occur in UK food products. The aim of this study was not to prove effects at real everyday food concentrations .The principle hypothesis under test was that it would be possible to develop metabolic markers of exposure and effect (relevant for assessment in higher mammals) that may, in future, enable early and reliable detection of systemic responses and health effects arising from such exposures.

2. Material and Methods

2.1. Pesticide selection

Compounds were selected for this study on the basis of having; a) been shown to occur in the UK food supply, b) specific, but differing modes of action and c) some evidence of

exceedance of the Acceptable Daily Intake (see section 2.2) in foodstuffs. The pesticides chosen were 2 benzimidazole fungicides; specifically carbendazim (CBZ) and thiabendazole (TBZ), and 2 bipyridyllium herbicides; specifically chlormequat (CMQ) and mepiquat (MPQ). These compounds are some of the most commonly found compounds in fruit, vegetables and cereals in national and co-ordinated monitoring programmes within the UK and Europe where doses above the ADI are sometimes reported (European Union, 2006).

2.2. Dose Selection

Since the aim of the study was to detect metabolic responses before permanent physiological changes manifested the pesticide exposure levels were chosen to be high enough to cause a metabolic response but not to result in overt clinical signs of toxicity. The exception to this is the lowest dose of each compound where it was decided to use a dose that was relevant to the human exposure level, assessed via the human Acceptable Daily Intake (ADI) as used by the UK Food Standards Agency (FSA). The ADI is a measure of the quantity of a particular chemical in food that can be consumed on a daily basis over a lifetime without harm. Theoretically this level of exposure should have no effect. All ADIs were taken from the latest edition of the pesticide manual (Tomlin, 2009) and the final dose ranges used in this study are outlined in table 1 and were approved by the FSA. The aim of the experiment was not to study the toxicological effects of these pesticides, which are already well studied (World Health Organization, 2005) but to investigate if metabolomics could be used give an early warning of effect at lower exposure levels than organ weight and feeding/drinking rates. Since the doses were not intended to relate directly to average food concentrations (but rather to determine if metabolomics could be used as early and sensitive detection of intakes exceeding ADI) they were selected between the ADI and levels causing any overt effects.

TABLE 1 TO BE INSERTED ABOUT HERE.

2.3. Animal handling and dosing

All experiments were approved by CEH ethics committee and conducted in accordance with the Home Office Guidelines for the Care and Use of Laboratory Animals (UK), which complies with the code of ethics of the world medical association (Declaration of Helsinki) for experiments involving animals.

Male Fischer F344 rats (8 weeks old) were obtained from Harlan (Bicester, UK) and maintained in constant conditions (21^{o}C, 12 hours light/12 hours dark) for 5 days prior to use in the study. During this period they were housed in groups in plastic cages with wood shavings as bedding material and had access to food (standard rat maintenance diet - RM1) in pellet form (Special Dietary Services; Witham, UK) and tap water *ad libitum*.

At the start of each exposure rats were randomly selected and weighed. The mean start weights were 200g +/- 1.45 SD (194-206g). They were then placed individually in metabolism cages where they again had free access to food (ground form of RM1) and water at all times. After 24 hours they were given a single dose, via oral gavage, of one of four doses of pesticide, or sham dosed as controls. The dosing vehicle was corn oil for the benzimidazole fungicides and distilled water for the bipyridyllium herbicides. Sham dosed animals were given just water or corn oil. The volume of each dosing vehicle was 0.5-1ml in all cases. Over the next five days urine samples were collected using metabolism cages. Thus, for each individual rat, urine was collected at a total of four time intervals, namely; the

24 hours before dosing (pre-dose) as well as 0-8, 8-24 and 96-120 hours post dosing. It is possible that any biomarkers observed using this method could be be related to the actual consumption of the tested compounds and not specific with toxicity. However, this is unlikely since the controls (dosed with water/corn oil) did not respond in the same away as the animals dosed with pesticides.

The rats were weighed for a second time at the end of each experiment to determine their weight gain over the 5-day testing period. Food and water consumption were also measured for each period the rats were in the metabolism cages. At the end of the experiment the rats were sacrificed using cervical dislocation and the liver, kidneys, brain and testes were removed and weighed (see figures 1-3).

2.4. Experimental Design

Due to availability of metabolism cages only 15 rats could be monitored individually at any one time. Rats were therefore split in batches and exposures staggered over 8 weeks, with both compounds from each pesticide class run at the same time (week 1, 2, 5 & 6 for CBZ/TBZ and week 3, 4, 7 & 8 for CMQ/MPQ) with common controls used for each pesticide class. Sodium azide was added to the urine collection pots to minimise bacterial degradation and all samples were stored at -80 ^{o}C immediately after collection.

2.5. ^{1}H-NMR analysis

All urine samples were analysed using ^{1}H NMR spectroscopy. Prior to NMR analysis, samples were thawed at 4 ^{o}C, spun at 447 g for 5 minutes and the supernatant collected. A

200 µl sub-sample of urine was then added to 400 µl of phosphate buffered deuterium oxide. The buffer consisted of 200 mM sodium phosphate, pH 7.4, containing 0.1 % sodium azide (to minimise bacterial degradation) and 1.2 mM sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) (Cambridge Isotope Laboratories Inc., Hook, UK) to provide a chemical shift reference ($\delta = 0.0$) for the resulting spectra. This mixture was then centrifuged for 10 minutes at 704 g and all samples were then transferred to individual 5 mm NMR tubes (Sigma-Aldrich Gillingham, UK) prior to analysis (Griffin et al., 2007).

Samples were analysed using a 500 MHz AVANCE II+ spectrometer (Bruker, Coventry, UK) operating at 500.13 MHz for the ^{1}H frequency using a 5 mm Broadband TXI Inverse ATMA probe. Spectra were collected using a solvent suppression pulse sequence based on a 1 dimensional NOESY pulse sequence to saturate the residual ^{1}H water signal (relaxation delay = 2 s, t_1 = 3 µs, mixing time = 150 ms, solvent presaturation applied during the relaxation time and the mixing time). 128 transients were collected into 16 K data points over a spectral width of 12 ppm at 27 ºC.

NMR spectra were processed using ACD SpecManager 1D NMR processor, version 8 (Advanced Chemistry Development Inc., Toronto, Canada) and Chenomx NMR Suite Professional, version 5.1 (Chenomx, Alberta, Canada). Free Induction Decays (FIDS) from the NMR were multiplied by an exponential weighting function equivalent to 1 Hz line broadening and then Fourier-transformed from the time to frequency domain. The resulting spectra were then manually phased, baseline-corrected, and referenced to the TSP singlet at 0 ppm. The spectra were converted into numerical vectors, representing the individual metabolites, by integrating across the spectrum using 0.004 ppm integral regions (bins). To account for any mass differences between samples, the binned intensities were normalized to

the total integral region. Individual integrals were thereby standardized to the total integral of all low weight molecular metabolites. Areas of the spectrum less than 0 ppm and greater than 9.5 ppm were removed since they contained only noise. The region between 4.5 and 5 ppm was also excluded in order to avoid the residual H_2O and urea peaks, since these are regions of the spectra are highly variable and contain little useful information (Beckwith-Hall et al., 1998).

Resonances in the spectra were assigned by the use of urine samples spiked with known standards, both for endogenous, biological metabolites and the pesticides metabolites (the latter obtained from B. Maddison, ADAS, Wolverhampton, UK) (Berger and Braun, 2004). Reference was also made to the library and peak fitting routine contained within the Chenomx software and the previous literature (Bollard et al., 2001; Connor et al., 2004; Griffin et al., 2000; Lenz et al., 2004). The online Madison Metabolomics Consortium Database (http://mmcd.nmrfam.wisc.edu/index.html) was also consulted. Assignments were also assessed by the use of two-dimensional NMR spectroscopy (COSY, HSQC, HMBC, JRES, and TOCSY pulse sequences).

2.6. Multivariate statistical analysis

Areas of the spectra associated with pesticide residues or pesticide metabolites were excluded from further analysis. The remaining data were Pareto scaled and analysed using two multivariate statistical techniques; Principal Components Analysis (PCA) and Partial Least Squares (PLS), using SIMCA-P software (version 11, Umetrics, Umea, Sweden). Identification of major metabolic perturbations within the pattern recognition models was achieved by analysis of the corresponding loadings plots. Additionally, R^2 and Q^2 were used

as measures for the robustness of the pattern recognition models (Eriksson et al., 1999). To examine dose responses and time trends the regression based PLS approach was also used to regress metabolic profile (X-block) against either dose or time (Y-variable). Following the completion of the studies and processing of the data the individual urinary metabolites found to significantly change in exposed animals compared to controls due to exposure to individual pesticides were identified (table 1).

3. Results

3.1 Physiological Changes

There were no observed gross physiological changes. All rats looked and behaved as normal during the study and analysis of tissues *post mortem* did not indicate any histopathological changes. Pesticide exposure did not result in any significant changes in weight gain (figure 1), feeding rates (figure 2), or organ (liver, kidney, brain and testis) weights (figure 3). This is shown by the high standard deviation in the data and was further assessed via paired t-tests ($p = >0.05$).

INSERT FIGURE 1-3 HERE.

3.2. NMR Analysis

NMR analysis of the urine samples resulted in robust and reproducible, high quality spectra (figures 4). Approximately 30 individual compounds could be identified. Prominent resonances included 2-oxo-glutarate, creatine and phenylacetylglycine. Observable metabolic

categories included amino acids (e.g. leucine, isoleucine and valine), carbohydrates (e.g. glucose), products of glycolysis (e.g. lactate), TCA cycle intermediates (e.g. succinate) and organic acids (e.g. formate). Pure samples of the pesticides and pesticide metabolites were also reliably measured by NMR.

FIGURE 4 INSERTED ABOUT HERE

3.3. Multivariate Analysis

To monitor the metabolic changes induced by exposure to the pesticides across the time course of the experiment PCA plots (figures 5) were created for each pesticide class. These models indicate that there were no metabolic changes in the control dosed animals (i.e. those that received corn oil or water only) but that each pesticide elicited a clear metabolic effect after dosing, but the extent and duration of this effect varied between compounds. For CMQ and MPQ the effect was detected after 0-8 hours with a return towards normality by 8-24 hours. In the case of CBZ and TBZ the time course was slower, with some metabolic responses not reaching their greatest extent until after 8-24 hour time point. In the case of TBZ some changes were still visible after 96-120 hours at the highest dose level.

The difference in the timing of the metabolic effect between compound classes may be attributable to the dosing of CMQ and MPQ in water and CBZ and TBZ in oil. The limited water solubility of CBZ and TBZ may also have resulted in a delay in their absorption profiles in the gut and thus their resulting efficacy (Hennessy, 1997). This is a common difficulty in comparing pesticides with very different solubility values. However, this is

unlikely to have had much of an effect since the control animals (dosed with water/corn oil) did not respond in the same away as the animals dosed with pesticides.

FIGURE 5 INSERTED ABOUT HERE

Figure 6 shows PCA plots with all doses and all time points for each compound. No effects were seen using the ADI for each compound but there was increasing response at the other dose levels. Although the time point of maximal response varied between the pesticide classes there was a clear dose response with the degree of departure from the controls which increased with dose and peaked at the highest dose for all 4 pesticides. For CMQ and MPQ this effect was only clearly observed at the highest dose level. In contrast, the results of TBZ and CBZ were more pronounced. For CBZ the two highest dose groups clearly separated from the majority of samples. The case of TBZ the effect was even stronger, with the three highest dose groups clearly separating from the majority of samples.

FIGURE 6 INSERTED ABOUT HERE

Using the loadings plots from the PCA analyses in figure 6 for each compound at each time point and dose allowed the identification of the specific regions in the NMR spectra that changed in response to compound exposure, as well as the assessment of how these changes related to both the dose level used and the time after dosing. These results are outlined in table 2. Again, TBZ and CBZ produced the greatest number of metabolic changes compared to CMQ and MPQ. For TBZ, CMQ and MPQ the largest numbers of metabolites were altered in the 0-8 hour time point. This number decreased in the 8-24 hour time point and by the 96-120 hour time point there were no metabolic differences compared to controls for CMQ and

MPQ. For CBZ there were an equal number of metabolic changes found for 0-8 hours and 8-24 hours, and for TBZ some metabolic changes were still present 96-120 hours post dosing. None of the metabolic changes detected were consistent across all four compounds demonstrating the different modes of action between pesticide classes and individual compound classes.

TABLE 2 INSERTED ABOUT HERE

4. Discussion

In order to have relevance and use from a regulatory perspective (and to avoid interference from signatures of overt toxicity) this study specifically set out to investigate low dose effects of pesticides relevant to potential human exposure. Since some NMR signals were unidentified there is a risk that the insights of the metabolic alterations induced by these toxins were compromised. However we feel this unlikely. In addition the stated aim of the study was to ascertain if metabolomics based assessment of easily obtainable biofluids could be used to detect the effects of pesticide exposure more quickly and at lower dose levels than traditional end points (such as growth or weight loss). This was found to be possible. For CMQ and MPQ this effect was detected after 8 hours with a return to normality by 24 hours. For CBZ and TBZ effects were detected after 8 hours, but continued to be apparent at 24 hours. In all cases no metabolic effects were detected for any of the pesticides at 96-120 hour, although for TBZ there was still a slight separation between the highest dose and the controls, while the lower doses had also returned to the control situation. With further long-term studies, the results presented here could potentially enable the reliable detection of systemic responses and health effects arising from such exposures far earlier than current methods.

Multivariate statistical analysis of the NMR spectra of rat urine samples demonstrated clear differences between the metabolic profiles of individuals exposed to each pesticide with these effects being both dose and time dependent. We utilised the rat for this study since this species continues to be extensively used by researchers as a model organism for investigating the biology and pathophysiology of human disease. Whole organism research provides a better understanding of whole biological system and all the complexity this encompasses (including interactions between systemic metabolism and specific organ toxicity) as opposed to cell culture techniques which may miss this level of detail.

Similar metabolite changes were between the same class of pesticides (i.e., TBZ and CBZ; CMQ and MPQ). These changes were seen in all but the lowest dose of each compound and grew in magnitude with increasing dose (figure 6). However, the time course varied between the compound classes with recovery occurring faster after exposures to CMQ and MPQ than for CBZ and TBZ. Metabolite responses varied greatly both between pesticides and between time points for each pesticide. There were several metabolite changes specific to each of the four compounds, but there are a few that are common within the two pesticide classes i.e. between CMQ and MPQ and CBZ and TBZ, respectively. However, no metabolic changes were unique to the high doses across all pesticides. It is of course debatable if an increase/decrease in one metabolite or group of compounds is an adverse effect or simply an adaptation to exposure. However, the fact that consistent and distinct changes were seen for each compound acts as proof of principal to show that pesticide exposure from food can be assessed by metabolomics and this has potential in food safety assessment.

The bipyridyllium herbicides (MPQ and CMQ) were found not to induce as large a response as the benzimidazole fungicides (CBZ and TBZ), possibly reflecting their differing modes of

action. Bipyridyllium herbicides may exert there effects in different ways. Compounds such as diquat and paraquat inhibit photosynthesis. Their mode of action is well understood and is due to their cationic moiety (Hess 2000). In chloroplasts or other affected areas these herbicides are initially reduced to radical cations, which, in turn, give rise to oxygen containing free radicals. These attack the sensitive unsaturated lipids of the cell membrane by peroxidation, eventually leading to the loss of membrane function (Adam et al. 1990). An often overlooked feature of bipyridylium herbicides is the fact that they are quaternary ammonium salts. As in any salt, stoichiometry requires that the di-cations must have the equivalent of anions as counterparts (e.g. chloride or bromide) but thus far little attention has been paid to the role of the anions in pesticide toxicity.

It has been hypothesized that these anions (particularly bromides) will form strong acids *in vivo* and that this might be responsible for their toxic effects in animals (Cochemé and Murphy, 2008). This is unlikely to be the case in this study however, since low concentrations of mepiquat and chlormequat chloride were used and chloride ions are ubiquitous in living cells. In addition, unlike diquat and paraquat the major mode of action of mepiquat and chlormequat is via the inhibition of gibberellin synthesis. Gibberellins are plant growth hormones which do not occur in animals. Since gibberellins do not have a specific target receptor in mammals the effects observed in this study are likely to be a general response to toxicological insult rather than a compound specific response. Thus, the effects of MPQ and CMQ observed in the rats used this study are likely to be a general response to toxicological insult rather than a compound specific response.

In contrast, benzimidazole fungicides are specific inhibitors of microtubule assembly, and thus do have a specific target in all cells. They act by binding to and “capping” the tubulin

molecule, which is the building block of the intracellular skeleton in eukaryotes (Davidse, 1986). Microtubules compose the cell skeleton and the spindle fibres (which separate the chromosomes during mitosis and meiosis) and thus their inhibition interferes with nuclear division as well as with related cell functions such as intracellular transportation in all eukaryotes (Gosteli, 2009). Thus, CBZ and TBZ most likely may have a more specific effect (and thus be more toxic) in mammals than CMQ and MPQ and this would seem to be reflected in the results reported here.

Reassuringly for consumers, the metabolomic analysis did not reveal any changes in the metabolite profile at the allowable daily intake (ADI) of each compound indicating that said ADI are valid, at least for individual compounds. It should perhaps also be noted that while Carbendazim may be used as a fungicide in itself, it is also the degradation product of other fungicides, such as benomyl (European Union, 2006). It is usually analysed as part of the benomyl-group, which comprises three different compounds (benonyl, carbendazim and thiophanate-methyl). Field based studies may therefore find it extremely difficult to determine to which pesticide a Carbendazim residue relates. In additions some toxicants may show clinical manifestations in specific organs, and other effects may be the result of delayed damage or syndrome. Thus urinary analysis is not enough to indicate the specific accumulation and post metabolic pathological changes. This means that further research to assess if ADIs are still suitable when exposure to two or more pesticides are present and/or when organs metabolic changes are assessed would be of considerable interest.

5. Conclusions

The use of metabolomics in food safety assessment has great potential (Davies, 2010). Since a mammalian model was used, the identification of the biochemical changes that are essentially "early effects biomarkers" in biofluid samples (collected with minimal intervention and on a relatively short timescale) has evident potential for monitoring both the occurrence and effects of low level pesticide exposure in humans. As such, this study illustrates the potential of NMR based metabolomics to provide a rapid and cost-effective screening tool for monitoring for the early effects of pesticides at lower doses than those affecting classical endpoints such as growth, behaviour and overall food intake. CBZ and TBZ had greater metabolic effects and thus would seem to be more toxic (in rats at least) than CMQ and MPQ. The technique therefore has the potential to enhance the ability of regulatory authorities to fulfil their mission to protect public health in relation to food.

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Figure 1

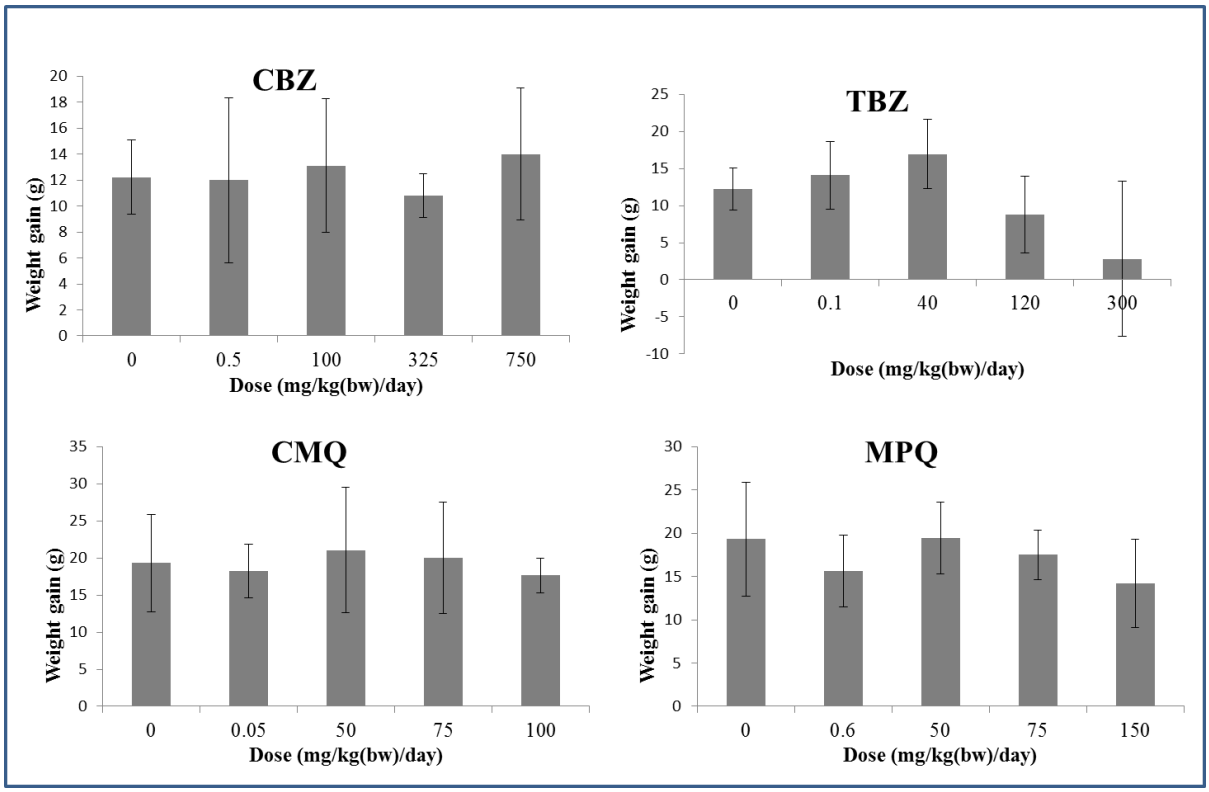
CBZ
Weight gain (g)
0
2
4
6
8
10
12
14
16
18
20
0
0.5
100
325
750
Dose (mg/kg(bw)/day)
TBZ
Weight gain (g)
-10
-5
0
5
10
15
20
25
0
0.1
40
120
300
Dose (mg/kg(bw)/day)
CMQ
Weight gain (g)
0
5
10
15
20
25
30
35
0
0.05
50
75
100
Dose (mg/kg(bw)/day)
MPQ
Weight gain (g)
0
5
10
15
20
25
30
0
0.6
50
75
150
Dose (mg/kg(bw)/day)

Figure 2

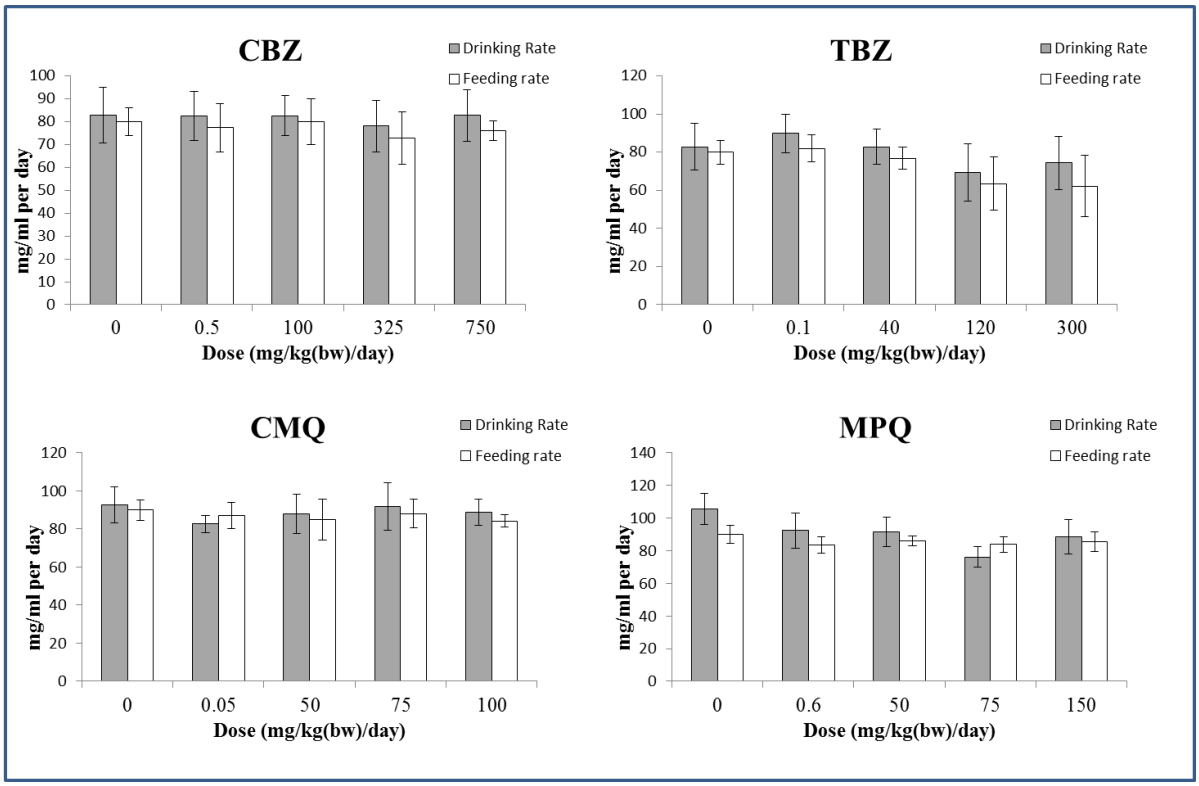
CBZ
Drinking Rate
Feeding rate
mg/ml per day
Dose (mg/kg(bw)/day)
0
0.5
100
325
750
TBZ
Drinking Rate
Feeding rate
mg/ml per day
Dose (mg/kg(bw)/day)
0
0.1
40
120
300
CMQ
Drinking Rate
Feeding rate
mg/ml per day
Dose (mg/kg(bw)/day)
0
0.05
50
75
100
MPQ
Drinking Rate
Feeding rate
mg/ml per day
Dose (mg/kg(bw)/day)
0
0.6
50
75
150

Figure 3

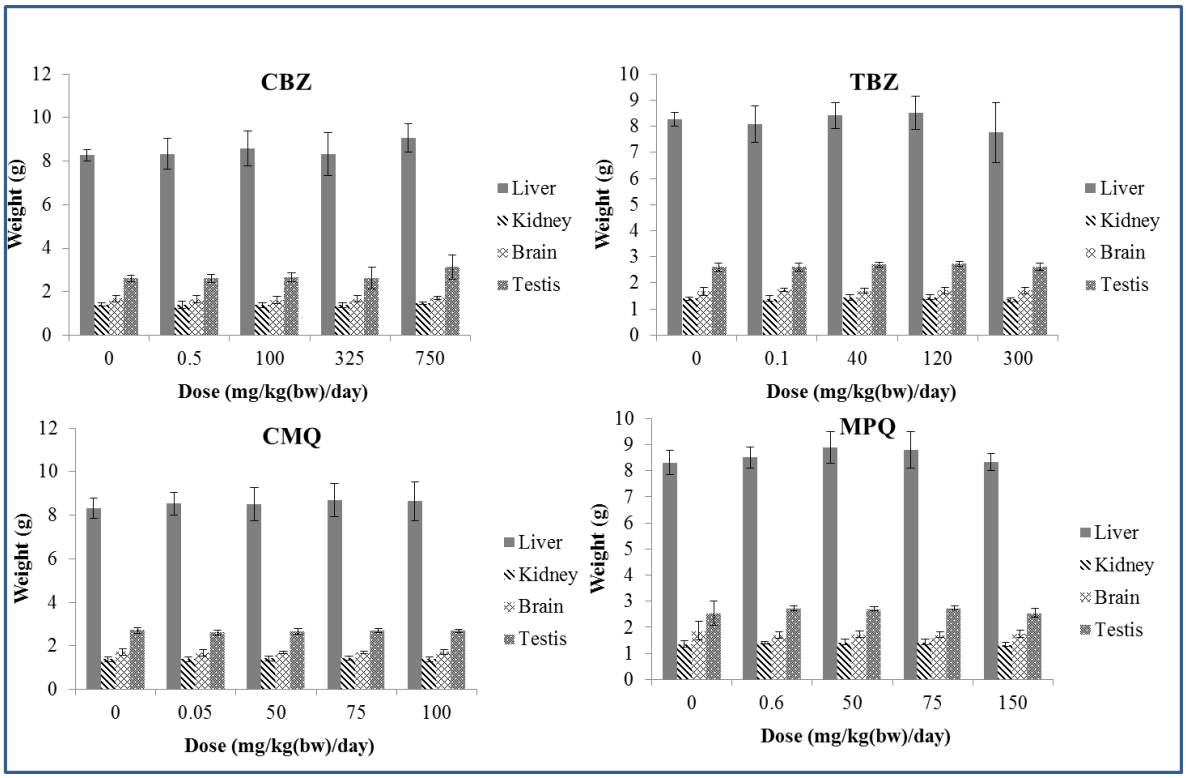
CBZ
Weight (g)
Dose (mg/kg(bw)/day)
0
0.5
100
325
750
TBZ
0
0.1
40
120
300
CMQ
0
0.05
50
75
100
MPQ
0
0.6
50
75
150
Liver
Kidney
Brain
Testis

Figure 4

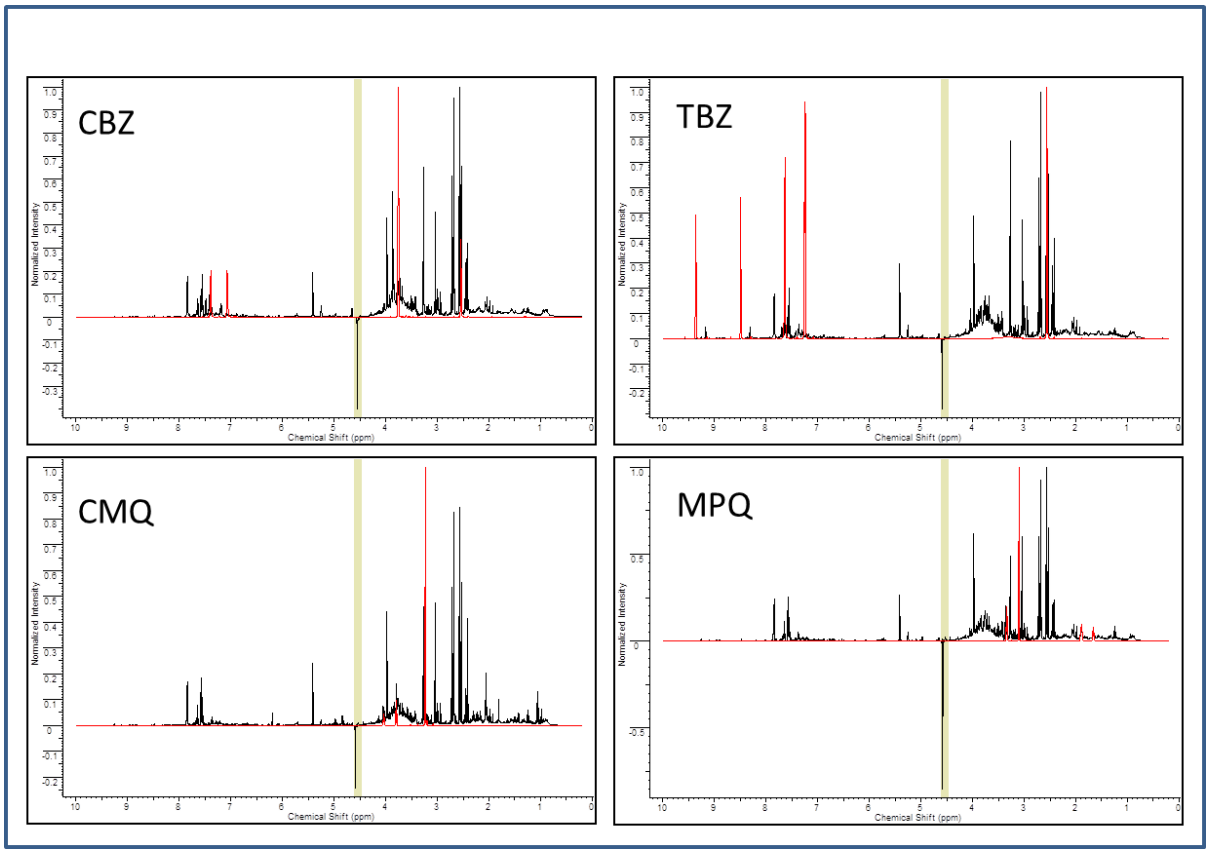

Figure 5

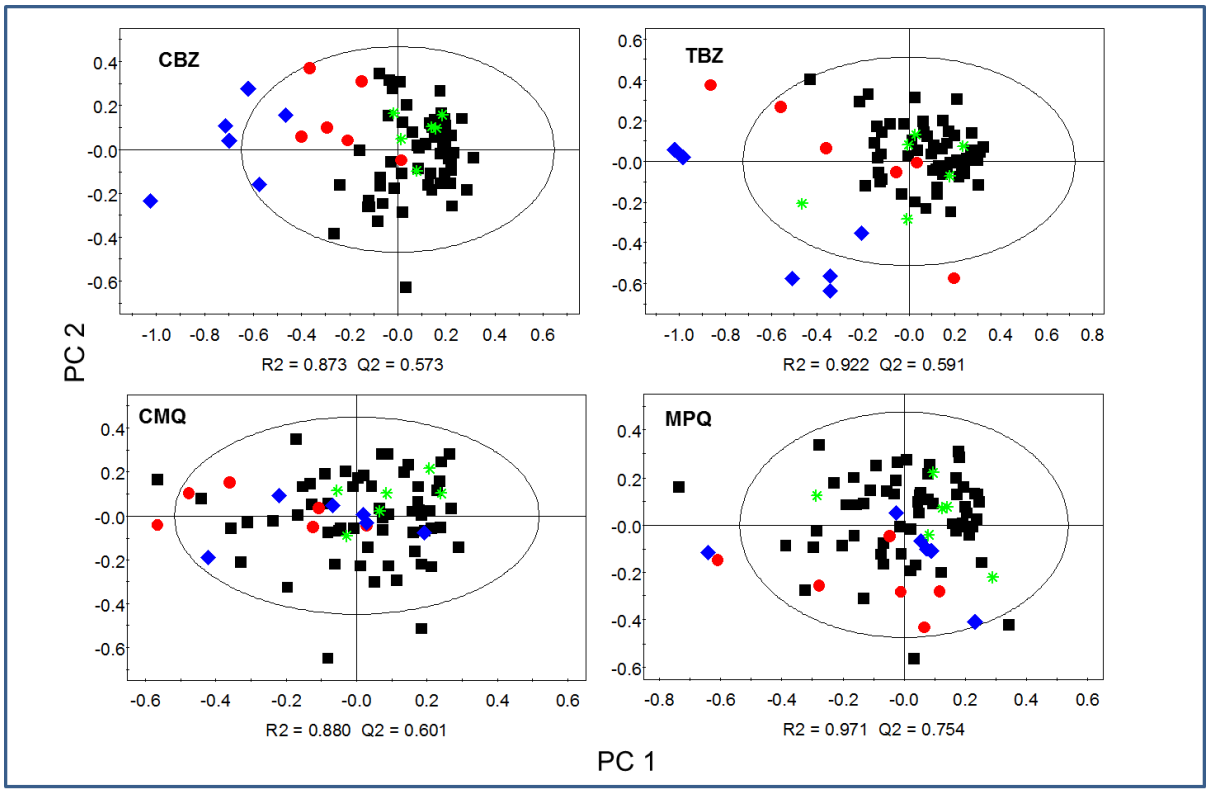
CBZ
R2 = 0.873 Q2 = 0.573
TBZ
R2 = 0.922 Q2 = 0.591
CMQ
R2 = 0.880 Q2 = 0.601
MPQ
R2 = 0.971 Q2 = 0.754
PC 2
PC 1

Figure 6

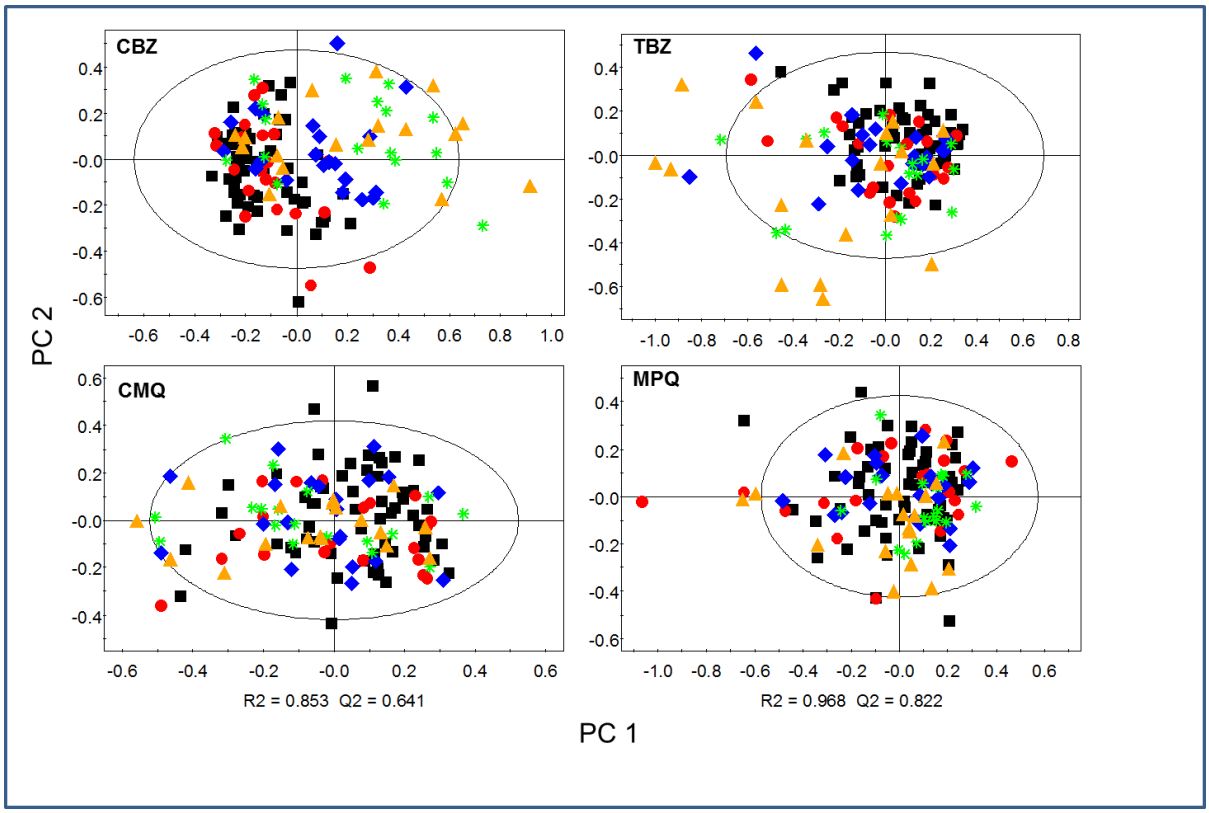

Table 1.

Pesticide	Low dose*	Intermediate dose 1	Intermediate dose 2	High dose
Carbendazim (CBZ)	0.5 (*200)	100 (*25 mg/kg(bw)/day*)	375	750
Thiabendazole (TBZ)	0.1 (*400)	40 (*9 mg/kg(bw)/day*)	120	300
Chlormequat (CMQ)	0.05 (*1000)	50 (*23 mg/kg(bw)/day*)	75	100
Mepiquat (MPQ)	0.6 (*83)	50 (*3000 ppm in diet ~ 240 mg/kg(bw)/day*)	75	150

* *Figures in parentheses indicate ratio of Intermediate dose 1/Low dose*
Figures in italics represent the no observed effect levels (NOEL) for each pesticide.

Low dose where it was decided to use a dose that was relevant to the human exposure level, assessed via the human (UK) Acceptable Daily Intake (ADI) as used by the Food Standards Agency (FSA) in the UK (see section 2.2).
Intermediate dose 1 – was aimed at being between 2 and 4 times higher than the estimated NOEL (given in italics in Table 1), which was achievable for CBZ, CMQ and TBZ. The only exception to this approach was for mepiquat as the suggested NOEL of 3000ppm seemed unreasonably high, and the low dose was that previously tested in a pilot study.

Intermediate dose 2 – was selected as a dose in between the high and intermediate dose 1 from which it was thought that changes in metabolism would result.

High dose – These doses set using pilot studies to be as high as reasonably possible without causing unacceptable signs of toxicity (e.g. a blinking response).

Table 2.

CBZ

Chemical Shift Range (ppm)	Metabolite	0-8hrs	8-24hrs	96-120hrs
2.40..2.42	b-hydroxybutyrate	—	▲	—
2.42..2.46	2-oxo-glutarate	▼	—	—
2.54..2.56	citrate	▼	—	—
2.66..2.68	aspartate	▼	—	—
3.02..3.06	creatine	▲	▲	—
3.26..3.28	betaine	▼	—	—
3.64..3.66	glycerol	—	▲	—
3.68..3.70	mannitol	—	▼	—
3.74..3.76	citrulline	—	▼	—
3.82..3.84	a-glucose	—	▲	—
3.84..3.86	fructose	▲	▲	—
5.38..5.40	unknown 1	▼	▼	—
7.02..7.04	unknown 2	—	▲	—
7.14..7.16	unknown 3	▲	▲	—
7.16..7.18	4-hydroxyphenylacetate	▲	—	—
7.38..7.40	phenylalanine	▲	—	—
7.40..7.42	phenylacetylglycine	▲	—	—
7.42..7.46	phenylalanine	▲	▲	—
7.52..7.54	nicotinate	▲	▲	—
7.60..7.66	hippurate	—	▲	—
7.80..7.82	uridine	—	▲	—
7.82..7.84	histidine	▲	—	—
7.84..7.86	hippurate	▼	▼	—

TBZ

Chemical Shift Range (ppm)	Metabolite	0-8hrs	8-24hrs	96-120hrs
2.42..2.46	2-oxo-glutarate	—	—	▼
2.46..2.48	2-oxo-glutarate	▼	—	—
2.48..2.50	unknown 4	▼	—	—
2.52..2.54	citrate	—	—	▼
2.66..2.68	aspartate	—	—	▼
2.92..2.94	S,S-dimethyl-b-propiothetin	—	—	▼
3.00..3.02	2-oxo-glutarate	—	—	▼
3.26..3.28	betaine	—	—	▼
3.62..3.64	fructose	—	—	▲
3.64..3.66	glycerol	—	—	▲
3.66..3.68	unknown 5	—	▲	—
4.02..4.06	gluconate	—	—	▲
4.14..4.16	proline	—	—	▲
7.20..7.22	tryptophan	▼	—	—
7.28..7.30	unknown 6	▲	▲	—
7.30..7.32	urocanate	▲	▲	—
7.32..7.34	phenylalanine	—	▲	—
7.52..7.54	nicotinate	▲	—	—
7.60..7.66	hippurate	▲	▲	—
7.66..7.68	4-aminohippurate	—	▲	—
7.68..7.72	4-aminohippurate	▲	—	—
8.28..8.30	unknown 7	▲	—	—
8.30..8.32	unknown 8	▲	—	—
9.14..9.18	unknown 9	▲	▲	—

CMQ

Chemical Shift Range (ppm)	Metabolite	0-8hrs	8-24hrs	96-120hrs
2.46..2.48	2-oxo-glutarate	▼	—	—
2.72..2.74	dimethylamine	▼	—	—
3.00..3.02	2-oxo-glutarate	▼	—	—
3.02..3.06	creatine	▼	▼	—
3.14..3.16	cystine	—	▼	—
3.22..3.26	carnitine	▲	▲	—
3.70..3.72	fructose_(b-furanose)	—	▼	—
3.74..3.76	citrulline	▼	—	—
3.78..3.82	arginine-phosphate	▲	▲	—
3.82..3.84	a-glucose	▼	—	—
3.88..3.90	mannitol	▼	—	—
3.90..3.92	fructose	▼	—	—
3.96..3.98	hippurate	▼	—	—
4.02..4.06	gluconate	▲	▲	—
5.38..5.40	unknown 1	▼	—	—
7.82..7.84	histidine	▼	—	—

MPQ

Chemical Shift Range (ppm)	Metabolite	0-8hrs	8-24hrs	96-120hrs
1.62..1.68	a-hydroxyisobutyrate	▲	▲	—
1.86..1.92	N-acetylglutamate	▲	▲	—
2.52..2.54	citrate	▼	—	—
2.70..2.72	N-acetylaspartate	▼	—	—
3.00..3.02	2-oxo-glutarate	▼	▼	—
3.02..3.06	creatine	▼	▼	—
3.08..3.12	cysteine	▲	▲	—
3.26..3.28	betaine	▼	—	—
3.32..3.36	unknown 10	▲	▲	—
3.74..3.76	citrulline	▼	—	—
3.96..3.98	hippurate	▼	—	—
5.38..5.40	unknown 1	▼	▼	—
7.54..7.56	hippurate	▼	—	—
7.82..7.84	histidine	▼	—	—